RECENT FINDINGS IN KYNURENINE RESEARCH IN THE FIELD OF EXPERIMENTAL EPILEPSY AND STROKE MODELS. ELECTROPHYSIOLOGICAL, BEHAVIOURAL AND HISTOLOGICAL STUDIES

Summary of Ph.D. Thesis

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Abbreviations

ACSF	artificial cerebrospinal fluid solution
AD	Alzheimer's disease
BBB	blood-brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
EAA	excitatory amino acid
fEPSPs	field excitatory postsynaptic potentials
FJ^+	Fluoro Jade B® positive
GABA	γ-aminobutyric acid
G-KYNA	glucoseamine-KYNA
HD	Huntington's disease
HFS	high-frequency stimulation
3-HK	3-hydroxykynurenine
IO	input-output
i.p.	intraperitoneally
KATs	kynurenine aminotransferases
KP	kynurenine pathway
KYNA	kynurenic acid
L-KYN	L-kynurenine
L-KYN+PROB-4VO	L-KYN + PROB pre-treated animals
LTP	long-term potentiation
MRPs	multidrug resistance-associated proteins
MS	Multiple sclerosis
NeuN	anti-neuronal nuclei
NMDA	N-methyl-D-aspartate
PD	Parkinson's disease
PROB	probenecid
PTZ	pentylenetetrazole
QUIN	quinolinic acid
SC	sham-operated controls
TRP	L-trypthophan
4VO	4-vessel occlusion
4VO-L-KYN+PROB	L-KYN + PROB post-treated animals

INTRODUCTION

L-tryptophan (TRP), one of the ten essential amino acids, is involved in protein synthesis and acts as a precursor of many biologically active substances. Besides its participation in the process of protein synthesis, in mammals TRP is metabolized in several pathways. The most commonly known is the serotonergic pathway, which is active in platelets and neurons, and yields 5-hydroxy-TRP and then serotonin. TRP is also the precursor of a pineal hormone, melatonin. A less well-known, but actually the main alternative route for the TRP metabolism, is through the kynurenine pathway (KP), a cascade of enzymatic steps involving a number of biologically active compounds. It is noticeable that 95% of the TRP is catabolised through the KP within the brain. The metabolites of the KP, collectively termed 'kynurenines', have been shown to take part in many diverse physiological and pathological processes. In particular, fluctuations in the levels of the kynurenines have discrete effects on the nervous and immune systems. Although a century has passed since the kynurenines were first recognized as major catabolic products of TRP, very little attention was paid to their possible involvement in biological processes until the 1980s.

The first KP metabolite of TRP, which was recognized as being neuroactive at the cellular level, was quinolinic acid (QUIN). This compound depolarises neurons by activating N-methyl-D-aspartate (NMDA) receptors. As a result, it is also able to produce excitotoxicity, and this realisation has led to QUIN being implicated in a variety of central nervous system (CNS) disorders.

The other important metabolite of KP is kynurenic acid (KYNA), which is one of the few known endogenous broad-spectrum antagonists of excitatory amino acid (EAA) receptors, especially the NMDA receptors. Kynurenic acid has been suggested to be involved in the pathophysiology of several brain disorders, including Parkinson's disease (PD), Huntington's disease (HD), Multiple sclerosis (MS), Alzheimer's disease (AD), stroke and epilepsy. In addition, accumulated data indicate that massively released excitatory amino acids play a major role in mediating the acute ischemic neuronal degeneration. Thus, one of the outstanding debates in the kynurenine field is, whether manipulation of the kynurenine pathway can modify the levels of kynurenic acid sufficiently to antagonize excitatory amino acid receptors. KYNA displays a particularly high affinity for the NMDA receptor, however, its therapeutic use is rather restricted, because KYNA has a very limited abilty to cross the blood-brain barrier.

L-kynurenine (L-KYN), a major component of the KP, serves as a source for the synthesis of all the other metabolites of the pathway. L-KYN is present in micromolar concentrations in the blood, brain and peripheral organs, and is transported through the blood-brain barrier (BBB) by the neutral amino acid carrier. L-KYN is further metabolized in three distinct ways. It serves as a substrate for kynureninase, for kynurenine aminotransferases (KATs) and for kynurenine-3-hydroxylase yielding, respectively, anthranilic acid, KYNA and 3-hydroxykynurenine (3-HK).

In the mammalian brain, KYNA is produced irreversibly from L-KYN by the action of the KATs, which are able to catalyse the transamination of L-KYN *in vitro*. Since excitatory amino acid receptor activation takes place in a variety of pathological states, KYNA has been tested as a neuroprotective agent.

Changes in the absolute and relative concentrations of the compounds involved in the KYN pathway (in particular KYNA and QUIN) in the brain have been implicated in a great number of neurodegenerative disorders, for example, stroke, epilepsy, ischemia, AD, HD, PD, MS and encephalopathies. Both agents are able to act on the NMDA-receptor complex and influence the glutamatergic transmission. Elucidation of the importance of the KP in the brain function has facilitated research relating to the alterations in the KP in various neurological disorders.

Hereinafter, we focused on epilepsy and ischemia from the above mentioned neurological disorders, because the accepted models of these two diseases were used in our works.

Epilepsy is one of the most common neurological disorders affecting 0.5-2% of the world's population. It varies widely in type and severity of seizures and should not be considered as a single disorder. It is currently defined as 'a tendency to have unprovoked recurrent seizures'. Epilepsy can result from brain injury caused by head trauma, stroke or infection, but in 6 out of 10 people seizures have no known cause. Seizures are the result of excessive neuronal firing temporarily disrupting neuronal signalling. This aberrant brain activity is the result of a shift in the balance between excitation and inhibition created by ion channels. In this context, it has been hypothesized that seizures and epileptogenesis may be due to either over-activation of excitatory pathways, utilizing glutamate or other excitatory amino acids, and/or a lesser activity of inhibitory pathways utilizing γ-aminobutyric acid (GABA) and other inhibitory neurotransmitters.

Pentylenetetrazole (PTZ), a chemical convulsant frequently utilized in the study of seizures, exerts its effects by binding to the picrotoxin-binding site of the post-synaptic GABA-A receptor. PTZ is known to suppress the inhibitory effects of certain neurotransmitters, and especially GABA, thereby leading to an easier depolarization of the neurons.

Glutamate receptors, especially NMDA receptors, are logical targets for new antiepileptic compounds. Unfortunately, early attempts at attenuating the function of these receptors by using conventional NMDA-receptor antagonists revealed serious side effects. For reasons that are not fully understood, these harmful consequences of direct NMDA-receptor blockade can be avoided by targeting the glycine co-agonist ($glycine_B$) site on the receptor for seizure suppression. Since glutamatergic neurotransmission plays a pivotal role in the pathogenesis of epilepsy, therefore antagonists of glutamate receptors are powerful anticonvulsants. In line with this, L-KYN, KYNA and their synthetic analogues are generally efficacious anticonvulsants in a variety of models of experimental epilepsy.

Stroke is the third major cause of death in the major industrialized countries after cardiovascular disease and cancer. The overall incidence of stroke is predicted to increase over the next decade by 12% but by around 20% in low-income families. Patients experiencing a typical large-vessel acute ischemic stroke will lose 120 million neurons, 830 billion synapses and 714 km of myelinated fibres each hour. Compared with the normal rate of neuron loss during aging, the ischemic brain will age 3.6 years for every hour the stroke goes untreated. The large majority (85%) of strokes in the western world are ischemic, that is, a stroke resulting from an occlusion of a major cerebral artery, commonly the middle cerebral artery (MCA) by a thrombus or embolism. The other strokes are hemorrhagic, where a blood vessel bursts either in the brain or on its surface. Ischemic brain injury results from a complex sequence of pathophysiological events that evolve over time and space. The major pathogenetic mechanisms of this cascade include excitotoxicity, peri-infarct depolarization and inflammation leading to cell death by apoptosis and necrosis. The main therapeutic approaches in patients with acute cerebral ischemia are thrombolysis and neuroprotection but none of them is really satisfying. Thrombolysis, which is aimed at restoring the cerebral blood flow, is restricted to few patients because of a narrow therapeutic window and a high hemorrhagic risk.

Transient global ischemia, which may arise during cardiac arrest and surgery in humans or be induced experimentally in animals, elicits selective, delayed neuronal death. If

the ischemia is short, neuronal damage occurs only in vulnerable areas. The pyramidal neurons in the hippocampal CA1 region are particularly vulnerable. Animal models of transient global ischemia, including bilateral carotid artery occlusion in Mongolian gerbils and 4-vessel occlusion (4VO) in rats, have demonstrated significant neuronal necrosis in the CA1 region of the hippocampus and impairments in a variety of learning and memory tasks. Other neurons, such as the hippocampal CA3 neurons, are less ischemia-vulnerable. It is widely accepted that activation of the EAA receptors plays an important role in neuronal death in stroke. It has recently been reported that glutamate-induced excitotoxicity and cellular calcium overload are among the key factors of cell death in brain ischemia, especially in the grey matter. In turn, neuroprotective strategies have utilized antagonists of the glutamate receptors to prevent excitotoxic neuronal loss.

Long-term potentiation (LTP), also mediated by glutamate receptors is a model of neuronal plasticity. Accordingly, ischemia may likewise impair physiological forms of synaptic plasticity, such as activity-dependent LTP. What is more, global ischemia may induce a special form of plasticity, anoxic LTP.

Impairments of the KP are strongly involved in neuronal death in various disorders. Prevention or correction of the abnormality, which results from changes in the levels of L-KYN derivatives, could attenuate the pathological processes.

At the moment, there appear to be three different possibilities for the development of therapeutic agents with the aim of modulation of the KP. One strategy is to use L-KYN as a precursor of neuroprotective KYNA. L-KYN can cross the BBB and increase the level of KYNA in the CNS. The second approach is to develop different pro-drugs and analogues of KYNA that can easily cross the BBB and act on the glycine-binding site of the NMDA receptors. The third method is manipulation of the KP by administration compounds that block the metabolism of L-KYN → QUIN conversion.

The following aims were set during our work on this topic:

To investigate

- whether L-KYN, which is able to cross easily the BBB by a neutral amino acid carrier and can be transformed into neuroprotective KYNA in the brain, can act against the acute PTZ-induced epileptic seizures?
- whether L-KYN administered intraperitoneally (i.p.) together with probenecid (PROB), (PROB is known to inhibit several transporters; among those are the multidrug resistance-associated proteins (MRPs), which act as organic anion

transporters), can be more effective than L-KYN administered alone and whether they are able to protect against the neurotoxic effect of PTZ?

- whether systemic pre- or post-treatment with L-KYN (administered together with PROB; i.p.) decreases the delayed cortical and hippocampal neuronal damage in rats subjected to transient global ischemia (4VO model)?
- whether our newly synthetized KYNA analogue glucoseamine-KYNA (G-KYNA) can readily pass into the brain after systemic administration (alone or together with PROB; i.p.) and can be more effective than KYNA?

MATERIALS AND METHODS

Animals

Our studies were performed on adult male Wistar rats (n=235, 250-300g) maintained under controlled environmental conditions at a temperature of 22±2 ˚C and 12-h light/dark cycle. Food and water were available *ad libitum*. Every effort was made to minimize animal suffering. The principles of laboratory animal care (NIH publication No. 85-23), and the protocol for animal care approved by the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed. In the *in vivo* electrophysiological experiments the summarized number of animals was 107, while 18 rats were used in *in vitro* electrophysiology. The number of animals starting the behavioural tests was n = 50. In the histological evaluation the summarized number of rats was 50, while 10 animals were used in the HPLC-MS/MS analysis.

Drugs

L-KYN (300 mg/kg; i.p.), PROB (200 mg/kg; i.p.), PTZ (60 mg/kg i.p.) and KYNA (17 μmol/kg) were obtained from Sigma (Steinheim, Germany), while our new compound G-KYNA (17 μmol/kg) (Fig. 3) was synthetized in the Institute of Medical Chemistry, University of Szeged. L-KYN, KYNA, G-KYNA and PROB was dissolved in 0.1 M sodium hydroxide (1ml) and adjusted with 1 M sodium hydroxide to pH 7.4.

In vivo electrophysiology

In the *in vivo* electrophysiologycal experiments we stimulated the CA3 area of the hippocampus and we recorded the hippocampal activity of contralateral CA1 area (rats were anaesthetized with urethane [1.3 g/kg, i.p.]). For recordings in area CA1, a 2-3 mm diameter hole was drilled over the dorsal hippocampus (3.0-3.8 mm posterior and 1.8-2.3 mm lateral to the sagittal suture) and the recording electrode was lowered 3.3 mm from the cortical surface.

Contralaterally, a 1-2 mm hole was drilled for the CA3 stimulating electrode (3.7 mm posterior to the bregma, and 3.3 mm lateral to the sagittal suture: final electrode depth 3.8 mm below the dura). Electrodes were lowered and final positions were adjusted so that the maximum CA1 population spike was obtained in response to contralateral CA3 stimulation. Responses to a range of stimulus intensities were recorded under control conditions to produce an input-output curve by changing the duration (10-100 μs), using current (up to 200 μA) square pulses. Stimuli were triggered at low frequency (0.05 Hz). Response stability was monitored for 30 min prior to drug administration. The electrophysilological recording continued during the following 3h recording period after drug administration.

Drug administration: On the basis of the literature data and our pilot experiments on the dose-dependent effects of these compounds, throughout the main studies L-KYN was administered in a dose of 300 mg/kg, PROB in a dose of 200 mg/kg, and PTZ in a dose of 60 mg/kg. After the pilot experiments in which the effects of the pure drugs were observed, we studied whether L-KYN+PROB inhibited the effects of PTZ. In these electrophysiological studies, the responses of area CA1 pyramidal cells to contralateral CA3 stimulation were tested for a 30-min control period. L-KYN and PROB administration followed. During the next 2 h, the low-frequency stimulation continued without recording, and PTZ was then administered. The electrophysiological effect was followed during a 3-h recording period.

Behavioural studies

In parallel with the *in vivo* electrophysiological experiments, behavioural studies were carried out on behaving animals. The behavioural effects of our neuroprotective compounds were studied in the water-maze and open-field tests. We were waiting 20 minutes after the treatments and began to do the water-maze performance. This examination was performed on five successive days. The open-field test was achieved on another day. From these results, we can conclude about the spatial learning, general locomotion and stereotype behaviour of the animals.

Four-vessel occlusion (4VO) model of transient global ischemia and drug treatment

The rats were anaesthetized with Nembutal (CEVA-PHYLAXIA; 60 mg/kg, i.p.). Both vertebral arteries were occluded by cauterization during careful cooling with iced washing. The wound were then closed, and the animals were allowed to recover for 24 h. On the following day, they were subjected to a 10-min forebrain ischemia by bilateral occlusion of the carotid arteries with atraumatic clips under ether anaesthesia. The body temperature

was monitored, and maintained at 37 ˚C during the surgical procedures, using a thermostatically controlled heating pad. Both vertebral arteries were cauterised, and both common carotid arteries were exposed, but not occluded in the sham-operated animals.

The rats used for cortical histology were divided into 5 groups: the group of control animals (n=5), which contain intact controls (IC) and sham-operated controls (SC); the 4VO group (4VO, n=7), the L-KYN + PROB pre-treated animals (L-KYN+PROB-4VO, n=6) and the L-KYN + PROB post-treated animals (4VO-L-KYN+PROB, n=7).

The rats used for hippocampal histology were divided into 4 groups: SC group (n=5), 4VO group (n=7), L-KYN+PROB-4VO group (n=6) and 4VO-L-KYN+PROB goup (n=7).

The rats used for *in vitro* electrophysiology were divided into 3 groups: SC group (n=6), 4VO group (n=6) and L-KYN+PROB-4VO group (n=6).

Drug administration: L-KYN (300 mg/kg, i.p.) and PROB (200 mg/kg, i.p.) were administered daily for 5 days: in the pre-treated group, the first L-KYN+PROB administration preceded the 10-min carotid occlusion by 2 h; and the animals were treated at the same time on the next 4 days. In the post-treated group, the animals received the first L-KYN+PROB injection at the start of reperfusion. The remaining 4 injections were given at the same time on the next 4 days.

Histological procedures

Ten days after 4VO, the rats were anaesthetized with urethane (1.3 g/kg, i.p.), and perfused transcardially with 0.1 M phosphate-buffered saline, and then with 4% paraformaldehyde. The brains were removed from the cranium, post-fixed in formalin, cryopreserved in 20% sucrose (containing 0.05% Na-azide), and sectioned at 32 μm with a cryostat microtome. The degenerating cells were stained with Fluoro Jade B®, while that of viable cells were identified by anti-neuronal nuclei (NeuN) immunohistochemistry. The locations of FJ-B positive (FJ^+) cells were observed with a fluorescence microscope (Olympus BX-51, Tokyo, Japan) with an excitation wavelength of 470-490 nm and an emission wavelength of 520 nm. The NeuN labelling was observed under a fluorescence microscope at an excitation wavelength of 530-550 nm, and an emission wavelength of 590 nm.

The global ischemia induced damage in both hemispheres, but in each case, the hemisphere with the greater extent of injury was evaluated. In case of cortical evaluation, the number of FJ^+ neurons/mm^2 was determined in each slide of each animal in the respective

group. A section was viewed and the injured neurons were counted at 4x magnification. In case of hippocampal evaluation, a section was viewed at 4x magnification and the most dorsal part of CA1 was chosen. Then, at 20x magnification, the image was captured with a 12-megapixel Olympus (DP-70) digital camera. The labelled cells were calculated for 1 mm^2. The numbers of FJ^+ and NeuN-immunopositive neurons/mm^2 were determined in each slide of all animals.

***In vitro* electrophysiology**

The electrophysiological recordings were conducted 10 days after 4VO. The rats (n=3x6) were decapitated, and coronal slices (400 μm) were prepared from the middle part of their hippocampi with a vibratome in an ice-cold artificial cerebrospinal fluid solution (ACSF) composed of (in mM): 130 NaCl, 3.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$, and 10 $_D$-glucose, saturated with 95% O_2 and 5% CO_2. The slices were then transferred into a Haas-type recording chamber and incubated at room temperature for 1 h to allow the slices to recover in the solution used for recording (differing only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$). The flow rate was 1.5-2 ml/min and the experiments were performed at 34 ˚C. The stimulating electrode was placed in the stratum radiatum near the border between the CA1 and CA2 regions to perform orthodromic stimulation of the Schaffer collateral/commissural pathway. Field excitatory postsynaptic potentials (fEPSPs) were recorded in parallel from the stratum radiatum and stratum pyramidale. The test stimulus intensity was adjusted to between 30 and 60 μA to evoke approximately 50% of a minimal stimulus intensity that evoked a saturated fEPSP (maximal fEPSP response) in SC rats.

LTP of the Schaffer collateral-CA1 synaptic response was induced by high-frequency stimulation (HFS; 0.2-ms pulses delivered at 100 Hz for 6 s) at 100% intensity of the test stimulus, and after HFS the fEPSPs were recorded for further period of at least 60 min. Input-output (IO) curves were created to measure the basal glutamatergic synaptic function. Slices from the same animal were generally used for both tests, including LTP and IO curves. Two slices were tested from each rat, and each slice was subjected to only one particular test.

HPLC-MS/MS analysis of L-KYN and KYNA levels in the plasma and brain tissue

The vertebral arteries were occluded 24 h before treatments with vehicle or L-KYN + PROB. Two hours later the rats were anaesthetized with pentobarbital (60 mg/kg, i.p.). No

bilateral carotid artery occlusion was performed. Blood was taken from the aorta, and centrifuged. After decapitation tissue samples were obtained from the hippocampus and cerebral cortex, and weighed. The plasma and brain tissue was stored at about -70 ˚C until analysis. Upon thawing, the brain tissue was homogenized in a potter. The HPLC-MS/MS system was used to measure the L-KYN and KYNA content of the samples.

Comparative *in vivo* electrophysiological examination of KYNA and G-KYNA

In these experiments, we studied the effects of peripherally administered G-KYNA on the hippocampal evoked activity, in comparison with peripherally administered pure KYNA. These drugs were administered alone, or in combination with PROB (200 mg/kg, i.p.). The doses of KYNA and G-KYNA that were chosen were based on earlier pilot experiments, in which both KYNA and G-KYNA were administered i.v. or i.p., in doses of 17, 34, 68 or 136 μmol/kg. Since i.p. and i.v. administration resulted in similar effects, i.p. administration was chosen in further studies. We endeavoured to find the minimum doses that were still effective; accordingly, on the basis of these pilot experiments, both KYNA and G-KYNA were administered in a dose of 17 μmol/kg throughout the main study.

Statistical analysis

Antidromic population spikes evoked by CA3 stimulation were measured from peak to peak. Differences between the amplitudes were determined statistically (paired *t*-test, *p* value set at 0.05 for significance). The results of behavioural experiments are expressed as means ± standard deviation (S.D.). Statistical analysis was performed by using the unpaired Student *t*-test or one-way analysis of variance (ANOVA) followed by LSD post-hoc test. Neuronal cell counts are presented as means ± standard error of mean (S.E.M.), and were analyzed by using one-way ANOVA followed by the Bonferroni test for multiple comparisons with SPSS version 9.0. A p value of ≤ 0.05 was considered significant. A nonparametric test on two independent samples was chosen for *in vitro* electrophysiological data (Mann-Whitney *U*-test).

SUMMARY OF OUR RESULTS

***In vivo* electrophysiology and behavioural studies**

In these experiments we examined whether pharmacological manipulations that lead to increased brain concentrations of KYNA could have any effects on the hippocampal electrical activity, and whether they have any antiepileptic effects. We decided to focus on the hippocampus, and on the question of whether co-administration of L-KYN together with

PROB enhances the effects of L-KYN. Accordingly, recordings were made *in vivo* and both behaviour and seizure susceptibility were examined, resulting in a more thorough study of the hippocampal function than if either of these features was studied alone. The evoked responses of hippocampal neurons were chosen as an end-point for these electrophysiological experiments because of the high concentrations of glutamate receptors on the dendrites of these neurons, and because they receive glutamatergic afferents that can be stimulated preferentially *in vivo*.

In our study, we administered a relatively high systemic doses of the precursor, L-KYN (in combination with PROB), which led to a non-physiological concentration of KYNA. This proved effective: L-KYN administered i.p. slightly (not significantly) decreased the population spike amplitude of the CA1 pyramidal cell responses. PROB had a somewhat stronger effect, with a longer delay. The two compounds administered together resulted in a marked and significant decrease in amplitude of the population spikes evoked on the CA1 pyramidal cells. This effect is probably based on the inhibition of the NMDA receptors of the CA1 pyramidal cells by the elevated level of KYNA in the brain tissue, as a consequence of peripheral L-KYN and PROB administration. This seemed to be able to compensate the overexcitation induced by PTZ. Indeed, 60 mg/kg PTZ administered i.p. resulted in a significant increase in amplitude of the CA1 spike activity, and this effect was completely compensated by pre-treatment with L-KYN+PROB. In parallel with the electrophysiological results, we studied the effects of L-KYN+PROB pre-treatment on the PTZ-induced seizures and death in awake animals. To test the possible protective effect of L-KYN+PROB in behaving animals, we chose the convulsive dose of 60 mg/kg PTZ. The pre-treatment with L-KYN+PROB was effective in protecting the animals against the generalized clonic seizures. Although they exhibited reduced rearing, washing and defecation activity, we did not observe any difference in behaviour between the controls and the treated animals in an open-field arena.

The primary finding of our study was that the peripheral administration of L-KYN and PROB eliminated the effects of PTZ; the responses to glutamatergic inputs were decreased in the CA1 region of the hippocampus, and in the behavioural experiments L-KYN and PROB pre-treatment protected the animals from the PTZ-induced kindling of seizures and death.

4VO model of transient global ischemia

Histology

It has long been known that a reduction in cerebral blood flow is closely related to different kinds of brain dysfunctions. The CA1 region of the hippocampus is particularly vulnerable to hypoxic conditions. Our results have shown that 4VO-induced temporal global ischemia results in neuronal damage not only in the hippocampus, but also in the neocortex. Moreover, L-KYN+PROB application significantly reduced this cortical neuronal damage after either pre- or post-ischemic administration. L-KYN+PROB administration considerably decreased the number of injured neurons in the CA1 region of the hippocampus. However, the decrease in the number of injured neurons was highly significant only in the pre-treated group. The animals in the post-treated group also exhibited a tendency to a reduction in the number of injured neurons, but this change was not significant. The NeuN immunohistochemistry supplemented these results: the number of non-injured cells was highest in the SC group, and lowest in the 4VO animals. Post-treatment with L-KYN+PROB had minimal effect, while in the 4VO animals, which received L-KYN before ischemia the number of intact cells was comparable to the control level.

The extensive literature suggests that an appropriate elevation of the brain KYNA concentration leads to a neuroprotective effect. The normal concentration of KYNA that we measured in the cerebrospinal fluid (CSF) by mass spectrometry is 20-30 nM. A considerable (800-1300-fold) increase in the brain KYNA level following the peripheral administration of L-KYN+PROB was demonstrated more than 15 years ago.

***In vitro* electrophysiology**

The input-output (IO) curves of SC, 4VO and 4VO animals which received L-KYN+PROB treatment were established by plotting the fEPSP amplitudes against various test pulse intensities from 0 to 100 µA. No significant difference was found between the IO curves in the three groups, implying that the basal functions of the pyramidal cells and synapses were not affected by complete ischemia. LTP was induced by HFS of the Schaffer collateral-CA1 synapses. In the SC group, the HFS caused a robust increase (170-180%) in the slope of the fEPSPs and this increase in slope (and in amplitude) remained at the elevated level during the 1h registration period. The same conditioning protocol did not induce significant, lasting increase of the fEPSPs in the majority of the 4VO animals. In this group, the elevation of the amplitudes was only transient; no LTP was observed. At the end of the registration period, the slopes had returned to the control level, or decreased below the baseline.

A novel finding here is that the administration of L-KYN+PROB once before and 4 times after 4VO-induced transient global ischemia proved neuroprotective in histological studies, and also reduced (nearly abolished) the impaired LTP induction in the Schaffer collateral - CA1 pathway in adult rats. In the majority of the 4VO animals, no LTP was observed. The administration of L-KYN+PROB protected the brain slices from the 4VO-induced LTP impairment. L-KYN restored the fEPSP slopes to the control level, and these parameters were stable until 60 min after HFS.

In most cases, after transient global ischemia, the surviving neurons displayed normal transmission, except for the reduction in the maximum level of fEPSPs that seems to be a consequence of the cell number reduction due to ischemic cell death. The impaired LTP induction should reflect deficits in the machinery specific to LTP induction in the individual surviving neurons. Although the mechanism is not yet known, we have demonstrated for the first time here that treatment with L-KYN+PROB rescues the Schaffer collateral-CA1 synapses from impaired LTP induction after transient global ischemia.

Plasma and brain L-KYN and KYNA concentrations

Treatment with L-KYN+PROB considerably increased both the L-KYN and KYNA concentrations in the plasma and brain, and also altered their proportions within the compartments studied, but this was probably due to the relatively large scatter of the data. In the sham-operated rats, L-KYN concentration was approximately 3-fold higher in the plasma than in the cortex and hippocampus. On the other hand, the KYNA concentration was higher in the cortex and hippocampus than in the plasma ($p<0.001$), and higher in the cortex than in the hippocampus. The L-KYN concentration increased 37-fold in the plasma and approximately 70-fold in the hippocampus and cortex, while the KYNA concentration was elevated roughly 300-fold in the plasma and 50-fold in the hippocampus and cortex.

Comparative *in vivo* electrophysiological examination of KYNA and G-KYNA

The primary finding of our comparative *in vivo* electrophysiological examination of KYNA and G-KYNA was that the peripheral administration of G-KYNA in a dose as low as 17 μmol/kg, especially together with PROB, effectively reduced the responses to glutmatergic input in region CA1 of the hippocampus, while pure KYNA injected either equimolarly or in higher doses, did not do so. These results suggests that the manipulation of brain kynurenines, e.g. increase of the KYNA level in the brain, may reduce the overactivation of EAA receptors, and offers novel therapeutic opportunities.

CONCLUSION

The discovery of the importance of the family of kynurenines in the brain function under physiological and pathological conditions has led to the development of powerful new compounds that promise to emerge as some of the first effective treatments for brain neuroprotection and as valuable adjuncts or alternative therapies for other CNS disorders. It is likely that, with continued development, a selection of new agents will soon be suitable for therapeutic intervention in the prevention or treatment of brain damage and neurodegenerative disorders, areas that at present remain almost impossible to treat.

Three potential therapeutic alternatives appear to offer the perspectives for the future (the use of pro-drugs of KYNA, like L-KYN; application of KYNA analogues; and manipulation of the KP by enzyme inhibitors). Nevertheless, it would be especially important to explore the L-KYN metabolism with softer and more sensitive methods in patients with different diseases. A good number of researchers have measured the levels of KYNA and QUIN in the plasma and CSF in certain conditions, but the causes and effects between KYNA and diseases are still not known in detail, and in most cases they are quite unknown. In the future, it would be useful to elicit the relationships between the KP and diseases through the application of sensitive molecular techniques in line with the development of powerful new compounds. In the face of all these challenges, it is clearly necessary to develop and spread the personal therapies of the patients with utilization of the recent research results.

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Scientific publications of the author of the Ph.D. thesis

Original papers directly related to the thesis

I. Németh, H., **Robotka, H.,** Kis, Z., Rózsa, É., Janáky, T., Somlai, C., Marosi, M., Farkas, T., Toldi, J. and Vécsei, L. (2004) Kynurenine administered together with probenecid markedly inhibits pentylenetetrazol-induced seizures. An electrophysiological and behavioural study. *Neuropharmacology*, **47**: 916-925.

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Total impact factor: 12.845

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